10/069575, 061202
JC10 Rec'd PCT/PTO 27 FEB 2002

TRANSMITTAL LETTER OT THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	Attorney Docket No **0508-1001** U.S. Application No **10/069575**

INTERNATIONAL APPLN. NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/08157	**22 AUGUST 2000**	**30 AUGUST 1999**

TITLE OF INVENTION: **HUMANIZED BIOMATERIALS, A PROCESS FOR THEIR PREPARATION AND THEIR APPLICATIONS**

APPLICANT(S) FOR DE/EO/US: **JACQUES BARTHOLEYNS**

Applicant herewith submits to the United States Designated Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31)
5. ☒ A copy of the International Application as filed (35 U.S C 371 (c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau)
   b. ☐ has been communicated by the International Bureau. See attached PCT/IB/308.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2))
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U S.C. 154(d)(4)
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made, however, the time limit for making such amendments has NOT expired
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U S C 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement (IDS) w/PTO-1449 - ☐ Copy of IDS citations
12. ☐ Assignment Papers (cover sheet & document(s))
13. ☒ A FIRST Preliminary Amendment.
14. ☐ A SECOND or SUBSEQUENT Preliminary Amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application (35 U.S C 154(d)(4)).
20. ☒ Other items or information: PCT/IPEA/409, International Search Report, Application Data Sheet, Abstract of the Disclosure on a Separate Sheet

Y & T 2/12/02

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U.S. APPLICATION NO. 10/069575	INTERNATIONAL APPLN. NO. 22 **AUGUST 2000**	ATTORNEY DOCKET NO 0508-1001

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1)-(5):		
Neither international preliminary examination fee nor international search fee paid to USPTO and international Search Report not prepared by the EPO or JPO $1040.00		
International preliminary examination fee not paid to USPTO but International Search Report prepared by the EPO or JPO $890 00		
International preliminary examination fee not paid to USPTO but International search fee paid to USPTO $740.00		
International preliminary examination fee paid to USPTO but all claims did not satisfy provision of PCT Article 33 (1)-(4) $710 00		
International preliminary examination fee paid to USPTO and all claims satisfied provision of PCT Article 33 (1)-(4) $100 00		
ENTER APPROPRIATE BASIC FEE AMOUNT	$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration than ☐ 20- ☒ 30 Months from the earliest claimed priority date (37 CFR 1.492(e))	$ 130 00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	14 - 20 =	0	X $18.00	$	
Independent Claims	1 - 3 =	0	X $84.00	$	
MULTIPLE DEPEND CLAIM(S) (if applicable)			+ $280.00	$	
			TOTAL OF ABOVE CALCULATION -	$ **1,020.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½. +				$	
			SUBTOTAL =	$ 1,020 00	
Processing fee of $130 00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492Z(f)).				$	
			TOTAL NATIONAL FEE =	$ 1,020.00	
Fee for recording the enclosed assigned (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) $40 00 per property +				$	
			TOTAL FEES ENCLOSED -	$ 1,020.00	
				Amount to be refunded	$
				Charged	$

☒ A Check in the amount of **$1,020.00** to cover all fees is attached.

☐ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to Deposit account No. 25-0120 in the name of Young & Thompson, as described below. A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fee required under 37 C.F R §§ 1.16 or 1 17

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Date: **February 27, 2002**

Benoit Castel
SIGNATURE

Benoit Castel
NAME

35,041
REGISTRATION NO.

PATENT
0508-1001

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of: Jacques BARTHOLEYNS

Appl. No.: **NEW** Group:

Filed: February 27, 2002 Examiner:

For: HUMANIZED BIOMATERIALS, A PROCESS FOR THEIR PREPARATION AND THEIR APPLICATIONS

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

February 27, 2002

Sir:

The following preliminary amendments and remarks are respectfully submitted in connection with the above-identified application.

IN THE CLAIMS:

Please amend the claims as follows:

3. (amended) Humanized biomaterial according to claims 1, wherein the human macrophages are liable to be obtained by *ex vivo* differentiation from blood monocytes leading to living macrophages, and are cultured under conditions enabling their penetration and adherence into the biomaterial, for instance for several hours at 37°C, with the porous biomaterial, allowing infiltration of the biomaterial and substantially irreversible binding of the living macrophages to the

biomaterial, being humanized with patient's macrophages and ready for implantation.

4. (amended) Living body-supporting implant, characterized by the fact that it comprises or consists of the humanized biomaterial according to claim 1, and is preferably structured under the form of scaffold, tissue-supporting sponges, bone or cartilage.

5. (amended) Use of a humanized biomaterial according to claim 1, for the preparation of a tissue implant destined to replace or repair defective tissue, such as defective bone, cartilage, dental tissue, fibrous tissue, fibrocartilaginous supporting tissue.

6. (amended) Use of a humanized biomaterial according to claim 1, wherein the monocyte derived cells or macrophages are autologous with respect to the tissue to be replaced or repaired, enabling the biomaterial or the living body-supporting implant to be recognized as self.

7. (amended) Process for the preparation of a humanized biomaterial according to claim 1, comprising the following steps:

- preparation of the porous biomaterial structured in form of bones, cartilage,

- preparation of macrophages from blood monocytes,

- immersion of the biomaterial in a physiologic solution appropriate for the culture of macrophages which are added

afterwards (ex. : phosphate buffered saline, medium such as RPMI, IMDM, AIMV),

- addition of the macrophages to the solution under conditions enabling binding to biomaterial and particularly for 1 to 20 h. at 37°C,

5% CO2 and 5% air,

- washing of the biomaterial and conservation until use in physiologic medium.

8. (amended) Process for the preparation of a living body-supporting implant according to claim 4, comprising the following steps:

- preparation of a customized porous implant or scaffold composed of bio-compatible material,

- preparation of macrophages from blood monocytes of the patient needing the customized implant of biomaterial,

- co-culture of macrophages and the implant in adequate medium under conditions enabling penetration and adherence to the biomaterial in particular at 37°C, 5% CO2 in hydrophobic bags or containers until grafting the implant.

9. (amended) Use of the humanized biomaterial according to claim 1, which can be implanted in a tissue, for the *in vitro* or *in vivo* or *ex vivo* delivery of factors chosen in the group of chemokines and/or monokines, and/or cytokines and/or growth factors, the factors released being useful for the local attraction of cells required for tissue growth (such as

osteoblasts, chondrocytes, fibroblasts, epithelial cells.....) and/or for the neovascularization around the implanted biomaterial, and/or for the release of growth factors sustaining proliferation of cells and/or the growth of new tissues.

10. (amended) Use of the humanized biomaterial according to claim 1, as a graft for the replacement of supporting tissues such as bones, cartilages, dental tissues, epithelial sheet and subcutaneous tissue matrix.

Please add the following claims:

--11. (new) Use of a living body-supporting implant according to claim 4, for the preparation of a tissue implant destined to replace or repair defective tissue, such as defective bone, cartilage, dental tissue, fibrous tissue, fibrocartilaginous supporting tissue.--

--12. (new) Use of a living body-supporting implant according to claim 4, wherein the monocyte derived cells or macrophages are autologous with respect to the tissue to be replaced or repaired, enabling the biomaterial or the living body-supporting implant to be recognized as self.--

--13. (new) Use of a living body-supporting implant according to claim 4, which can be implanted in a tissue, for the *in vitro* or *in vivo* or *ex vivo* delivery of factors chosen in the group of chemokines and/or monokines, and/or cytokines

and/or growth factors, the factors released being useful for the local attraction of cells required for tissue growth (such as osteoblasts, chondrocytes, fibroblasts, epithelial cells.....) and/or for the neovascularization around the implanted biomaterial, and/or for the release of growth factors sustaining proliferation of cells and/or the growth of new tissues.--

--14. (new) Use of a living body-supporting implant according to claim 4, as a graft for the replacement of supporting tissues such as bones, cartilages, dental tissues, epithelial sheet and subcutaneous tissue matrix.--

REMARKS

Claims 1-14 are pending in the present application. Claims 11-14 have been added.

Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly requested.

Should there be any matters that need to be resolved in the present application, the Examiner is respectfully requested to contact the undersigned at the telephone number listed below.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE."

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

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BC/ia
Attachments

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS:

The claims have been amended as follows:

3. (amended) Humanized biomaterial according to claims 1~~ or 2~~, wherein the human macrophages are liable to be obtained by *ex vivo* differentiation from blood monocytes leading to living macrophages, and are cultured under conditions enabling their penetration and adherence into the biomaterial, for instance for several hours at 37°C, with the porous biomaterial, allowing infiltration of the biomaterial and substantially irreversible binding of the living macrophages to the biomaterial, being humanized with patient's macrophages and ready for implantation.

4. (amended) Living body-supporting implant, characterized by the fact that it comprises or consists of the humanized biomaterial according to ~~any one of claims 1 to 3,~~claim 1, and is preferably structured under the form of scaffold, tissue-supporting sponges, bone or cartilage.

5. (amended) Use of a humanized biomaterial according to~~any one of claims 1 to 3 or of a living body-supporting implant according to~~ claim ~~4,~~1, for the preparation of a tissue implant destined to replace or repair defective tissue, such as defective bone, cartilage, dental tissue, fibrous tissue, fibrocartilaginous supporting tissue.

6. (amended) Use of a humanized biomaterial according to ~~any one of claims 1 to 3 or of a living body-supporting implant according to~~ claim ~~4,~~1, wherein the monocyte derived cells or macrophages are autologous with respect to the tissue to be replaced or repaired, enabling the biomaterial or the living body-supporting implant to be recognized as self.

7. (amended) Process for the preparation of a humanized biomaterial according to ~~any one claims 1 to 3,~~claim 1, comprising the following steps:

- preparation of the porous biomaterial structured in form of bones, cartilage,

- preparation of macrophages from blood monocytes,

- immersion of the biomaterial in a physiologic solution appropriate for the culture of macrophages which are added afterwards (ex. : phosphate buffered saline, medium such as RPMI, IMDM, AIMV),

- addition of the macrophages to the solution under conditions enabling binding to biomaterial and particularly for 1 to 20 h. at 37°C,

5% CO2 and 5% air,

- washing of the biomaterial and conservation until use in physiologic medium.

8. (amended) Process for the preparation of a living body-supporting implant according to claim 4, comprising the following steps:

- preparation of a customized porous implant or scaffold composed of bio-compatible material, ~~according to any one of claims 1 to 3,~~

- preparation of macrophages from blood monocytes of the patient needing the customized implant of biomaterial,

- co-culture of macrophages and the implant in adequate medium under conditions enabling penetration and adherence to the biomaterial in particular at 37°C, 5% CO2 in hydrophobic bags or containers until grafting the implant.

9. (amended) Use of the humanized biomaterial according to~~any one of claims 1 to 3 or of a living body-supporting implant according to~~ claim ~~4,~~1, which can be implanted in a tissue, for the *in vitro* or *in vivo* or *ex vivo* delivery of factors chosen in the group of chemokines and/or monokines, and/or cytokines and/or growth factors, the factors released being useful for the local attraction of cells required for tissue growth (such as osteoblasts, chondrocytes, fibroblasts, epithelial cells.....) and/or for the neovascularization around the implanted biomaterial, and/or for the release of growth factors sustaining proliferation of cells and/or the growth of new tissues.

10. (amended) Use of the humanized biomaterial according to~~any one of claims 1 to 3 or of a living body-supporting implant according to~~ claim ~~4,~~1, as a graft for the replacement of supporting tissues such as bones, cartilages,

dental tissues, epithelial sheet and subcutaneous tissue matrix.

Abstract of the Disclosure

The invention relates to humanized biomaterial comprising a porous biocompatible composite material customized and implanted with monocyte derived cells preferably with macrophages.

NEW HUMANIZED BIOMATERIALS, A PROCESS FOR THEIR PREPARATION AND THEIR APPLICATIONS

The invention relates to new humanized biomaterials, a process for their preparation and their applications.

Tissue repair is needed after severe bone fractures, cartilage loss or general fragilization during ageing.

Artificial metallic or even ceramic prostheses are not very well integrated within host tissues and replacement with new surgery is often required after a few years, a major problem of handicap for old people.

Autologous grafts of bones or cartilage tissue is very difficult and costly. New technologies develop porous matrices implanted as scaffold prosthesis.

These matrices can eventually be filled with growth factors for the tissue regeneration or even with bone marrow stem cells.

However, fixation of cells or factors in the porous matrices with very prolonged and slow release of growth factors is very difficult to achieve and the ideal cocktail and concentration of factors required is unknown.

The aim of the present invention is to provide a homogeneous humanized, bioactive biomaterial (for example porous ceramics) that can be used for implantation purposes and which do not present the long term biocompatibility problems of prior art.

Another aim of the invention is to provide a bioactive biomaterial enabling tissue growth (for example bone and cartilage) in its porous space and securing the integration of the grafted biomaterial in the surrounding tissues (viable bones...).

Another aim of the invention is to provide long lasting prostheses, which avoids requirement for replacement of biomaterial prostheses after 10 years, as often needed up to now.

These aims are achieved by the invention, which consists in humanized biomaterial comprising a porous biocompatible composite material customized and implanted with monocyte derived cells and preferably with macrophages.

The expression "humanized" means that the porous biomaterial has been colonized with human cells derived from blood monocytes.

The expression "biocompatible composite" material designates a material composed of one or several of the following materials proved to be non toxic for human tissues (carbon microfibers, ceramics, calcium phosphates, metal oxides, collagen polymers...).

The expression "porous" means that the biomaterial and preferably the ceramic present pores of about 100 to 2000 microns of diameter.

The expression "customized and implanted" material means that the shape and size of biomaterial is designed specifically for a patient and a site of implantation.

The expression "monocyte derived cells" corresponds to human mononuclear cells isolated from blood, enriched in monocytes and cultured at 37° C in appropriate medium, for 5 to 10 days to obtain tissue type macrophages.

The monocyte derived cells used in the invention are for instance such as those described in PCT/EP 93/01232, WO 99/13054, EP 96/ 901848.0-2107, WO 97/44441.

In a particular embodiment of the invention, the monocyte derived cells described above, contain exogenous compounds such as drugs, proteins, growth factors of interest.

In another embodiment, the monocyte derived cells as described above contain in their cytoplasm exogenous DNA coding for a protein of interest.

The substantially irreversible humanization of matrices of biocompatible composite material described in the present invention allows a physiological interaction between the prostheses made of the biomedical composite, grafted and the host cells in the body. These relations with host tissue cells and with the extracellular matrix allow reconstruction of epithelial sheets and growth of a capillary network around the grafted biomaterial by local multiplication and sprouting of endothelial cells.

The monocyte derived cells, in particular the macrophages used to humanize *in vitro* the porous material in the invention are particularly adequate to increase integration and *in vivo* lifespan of biocompatible prostheses.

Advantageously, the humanized biomaterial of the invention is homogenous.

According to an advantageous embodiment, in the humanized biomaterial of the invention, the biocompatible composite material is chosen among the following materials : microfibres, ceramic materials, metal oxides such as aluminum oxide, calcium phosphate ceramic, glass or carbon fibers, hydroxylapatite, silicon carbide or nitride, collagen polymers or a mixture of these different materials.

According to another advantageous embodiment, in the humanized biomaterial of the invention, the human macrophages are liable to be obtained by *ex vivo* differentiation from blood monocytes leading to living macrophages, and are cultured under conditions enabling their penetration and adherence into the biomaterial for instance for several hours at 37°C, with the porous biomaterial, allowing infiltration of the biomaterial and substantially irreversible binding of the living macrophages to

the biomaterial, now humanized with patient's macrophages and ready for implantation.

The expression "substantially irreversible binding" means that macrophages are tightly bound by numerous contacts with the material and cannot be detached under physiological conditions.

The invention also relates to a living body-supporting implant, characterized by the fact that it comprises or consists of the humanized biomaterial according to any one of claims 1 to 3, and is preferably structured under the form of scaffold, tissue-supporting sponges, bone or cartilage.

The expression "living body-supporting implant" designates an implant having for example the physical form and robustness of a bone to be replaced.

The expression "scaffold" designates a physical structure eventually metallic which keeps the biomaterial (ceramics or other) in the appropriate conformation.

The expression "tissue supporting sponges" designates a soft implant formed for instance of collagen which will be humanized with macrophages before insertion in the body.

The invention also relates to the use of a humanized biomaterial of the invention or of a living body-supporting implant of the invention, for the preparation of a tissue implant destined to replace or repair defective tissue, such as defective bone, cartilage, dental tissue, fibrous tissue, fibrocartilaginous supporting tissue.

The expression "fibrous tissue" designates tissues surrounding organs which support this organ and maintain the shape of this body part : they are mainly formed by epithelial sheets.

According to another advantageous embodiment, the invention relates to the use of a humanized biomaterial of the invention or of a living body-supporting implant of the invention, wherein the monocyte derived cells or macrophages are autologous with respect to the tissue to be replaced or repaired, enabling the biomaterial or the living body-supporting implant to be recognized as self.

The expression "implant to be recognized as self" means that it contains cells (i.e. macrophages) from the patient in which it will be grafted and is therefore autologous to the host.

A process for the preparation of a humanized biomaterial of the invention comprises the following steps :

- preparation of the porous biomaterial structured in form of bones, cartilage,
- preparation of macrophages from blood monocytes,

- immersion of the biomaterial in a physiologic solution appropriate for the culture of macrophages which are added afterwards (ex. : phosphate buffered saline, medium such as RPMI, IMDM, AIMV),
- addition of the macrophage to the solution under conditions enabling binding to the biomaterial and particularly for 1 to 20 h. at 37°C, 5 % CO2 and 5 % air,
- washing of the biomaterial and conservation until use in physiologic medium.

A process for the preparation of a living body-supporting implant of the invention comprises the following steps :

- preparation of a customized porous implant or scaffold composed of bio-compatible material, according to any one of claims 1 to 3,
- preparation of macrophages from blood monocytes of the patient needing the customized implant of biomaterial,
- co-culture of macrophages and the implant in adequate medium under conditions enabling penetration and adherence to the biomaterial, in particular at 37°C, 5% CO2 in hydrophobic bags or containers until grafting the implant.

The invention also relates to the use of the humanized biomaterial or of a living body-supporting implant, which can be implanted in a tissue, for the *in vitro* or *in vivo* or *ex vivo* delivery of factors chosen in the group of chemokines and/or monokines, and/or cytokines and/or growth factors, the factors released being useful for the local attraction of cells required for tissue growth (such as osteoblasts, chondrocytes, fibroblasts, epithelial cells.....) and/or for the neovascularization around the implanted biomaterial, and/or for the release of growth factors sustaining proliferation of cells and/or the growth of new tissues.

Indeed, macrophages maintain tissue homeostasis through the secretion of at least 80 growth factors or monokines controlling and inducing proliferation of mesenchymal (fibroblasts....), endothelial, chondrocytes, osteoblasts, **epithelial** cells.. They also secrete enzymes and mediators allowing growth and renewal of the surrounding cells and tissues (see Table 1).

The key factors secreted by macrophages supporting tissue integration regeneration and growth of mesenchymal cells are : IGF1 and TGFs, but also PDGF, bFGF, MDGF, GM, CSF, NAF, IL-8, TNF, angiogenin and angiogenic factors. These growth factors allow also the development of all the steps required for angiogenesis, allowing neovascularisation and reconstitution of blood microcapillaries around the grafted biomaterial.

In this aspect, macrophages are synthesizing 10 fold more proteins than monocytes, much more growth factors and less inflammatory mediators.

TABLE 1 :

GROWTH FACTORS PROTEINS AND MEDIATORS SUPPORTING TISSUE HOMEOSTASICS SECRETED BY NATURE MACROPHAGES

ENZYMES :

Lyzosymes

Neutral proteases
- Plasminogen activator
- Collagenase
- Elastase
- Angiotensin-convertase

Acid hydrolases
- Proteases
- Lipases
- Ribonucleases
- Phosphatases
- Glycosidases
- Sulphatases

Arginase

COMPLEMENT COMPONENTS
C1, 4, 2, 3 and 5
Factors B and D and Properdin
C1 inhibitor
C3b inactivator and β-1H

ENZYME INHIBITORS
(Antiproteases)

α1-antiprotease
Plasmin inhibitors
α-2 macroglobulin
Plasminogen activator inhibitors

PROTEINS BINDING METABOLIOUS AND LIPIDS :
Acidic isoferritins

BIOACTIVE LIPIDS :

Arachidonic acid metabolites
- Prostaglandins E2, F2α
- Prostacyclin
- Thromboxane
- Leukotrienes B4, C, D and E
- Hydroxy-eicosatetraneoic acids (including SRS-A)

Platelet activating factors

CYTOKINES, HORMONES, GROWTH FACTORS :
- Interleukins 1 α and β
- Tumours necrosis factor α
- Interferons α and β_1, &
- Interleukin 6, 8, 13, 18

Chemotatic factors for
- Neutrophils
- Tlymphocytes
- Monocytes
- Fibroblasts

Heamatopoceitic Colony Stimulating Factors for
- Granulocyte-Macrophages (GM-CSF)
- Granulocytes (G-CSF)
- Macrophages (M-CSF)
- Erythropoeitin

Growth factors
- Fibroblasts growth factor / Insuline like G.F.
- "platgelet-derived growth factor" (PDGF)
- Transforming growth factor α and β
- Endothelial cell growth factor

Hormones
- 1 α, 25-Dihydroxyvitamin D3

Transferrin
Transcobalamin II
Fibronectin
Laminin
Lipid transfer protein
Thrombospondin

NUCLEOSIDES AND METABOLITES :
Thymidine and deoxycytidine
Uracil
Uric acid
Lactic acid
Polyamines nitrines and nitrates
Neopterin

Insulin-like activity, protagandins
Thymosin B4
β endorphin
Adrenocorticotrophic hormone

CHEMOKINES MIP / RANTE FAMILIES
COAGULATION FACTORS :
Factors III, VIII, and tissue factor
Prothrombin and prothrombinase
Factors IX, X, V and VII

In an advantageous embodiment of the invention, the macrophages migrate initially in the porous biomaterial and incorporate irreversibly into this prosthesis by very strong adherence and spreading. When they are kept in physiological conditions, macrophages are very long living cells lasting from several months to several years after implantation. During this time, macrophages will continuously secrete growth factors and cytokines in their local environment ; these factors will act in synergy at very low concentrations (10^{-10} M) on the surrounding cells and tissues.

Furthermore, macrophages do present on their membranes receptors for cytokines, hormones, sugars allowing to respond to micro-environmental needs and to adjust their secretion to the local status around the biomaterial at different periods after grafting.

The growth factors secreted by macrophages represent the global requirements for tissue repair, differentiation and local angiogenesis. The chemokines which will be continuously released in a concentration gradient around the implant will call in and around the prosthesis cells required for recolonization and integration of the biomaterial in host environment. Therefore, the new customized porous biomaterials colonized with host macrophages present a novel biotolerance and a length of adequate performance far better than prosthesis used in the absence of autologous macrophages. Applications are very large in solid or cartilaginous prosthesis needed in bone, cartilaginous repair particularly.

According to another advantageous embodiment, the invention relates to the use of the humanized biomaterial or of a living body-supporting implant, as a graft for the replacement of supporting tissues such as bones, cartilages, dental tissues, epithelial sheet and subcutaneous tissue matrix.

Example 1 :

A calcium phosphate porous ceramic with pores of 200 to 2000 microns (porosity > 20 % and < 80 %) is placed on an hydrophobic support (ethylene vinyl acetate) in the presence of 50 ml AIMV culture medium (life-cell Gibco, Paisley G.B.). Macrophages are added to this preparation at the concentration of 5.10^6 cells/ml ; they are obtained after 7 days differentiation of blood monocytes in culture according to published state of the art in publications and patents (PCT/EP 93/01232, WO 99/13054, EP 96/ 901848.0-2107, WO 97/44441) ; a control preparation is maintained in the absence of macrophages. The preparation is incubated overnight at 37 °C, 5 % CO2, 95 % air to allow fixation of macrophages on the ceramic.

The porous ceramic is washed and cultured in the presence of fibroblasts and/or in the presence of chondrocytes In both cases, the cell proliferation is higher by a factor 2 to 10 for the porous ceramic colonized with macrophages, compared to control ceramic.

Example 2 :

A small fragment of porous microceramic is implanted in a rabbit cornea. The inert microceramic piece induces a very small to moderate inflammation and only peripheral growth of new blood vessels from the ring of the cornea.

In contrast, microceramic covered with macrophages, as described in example 1 induces a major neovascularisation towards the center of the cornea. The cornea becomes vascularized through an invasion of endothelial cells arising from the rim rich in blood supply and sprouting towards the biomaterial implanted.

Example 3 :

Fragments of 100 +/- 20 mg of hydroxyapatite ceramic (Endobon®, Merck) and pieces of one cm2 of a polypropylene scaffold are prepared. Fresh non activated macrophages obtained after 7 days differentiation of blood monocytes in culture according to published patent applications (WO94/26875, WO 99/13054, WO96/22781, WO 97/44441) are suspended (2,5.106 cells/ml) in IMDM (Iscove Modified Dulbecco Medium) culture medium. Each biomaterial fragment is incubated in 1 ml of macrophage suspension, in sterile polypropylene tubes, for 4 hours at room temperature. To check the binding of macrophages on the biomaterial, cells present in the supernatant after incubation are counted. After incubation with Endobon®, from 12 to 17% of the cells added were present in the supernatant (3 experiments),

indicating that more than 2.10^6 macrophages are adsorbed on 100 mg of porous ceramic. After incubation with polypropylene scaffold, from 23 to 55 % of the cells initially added are present in the supernatant, indicating adsorption of 1 to 2.10^6 macrophages/cm2 scaffold.

Nude mice are implanted with biomaterial, and each mouse receives two implants of the same type, one in each flank.

Mice n°	Implanted material
1, 2	Endobon® colonized by macrophages
3	Endobon®
4, 5	Polypropylene colonized by macrophages
6	Polypropylene

Mice are sacrificed after 21 days, macroscopic observation shows no major difference between implants of biomaterial alone and implants of biomaterial colonized by macrophages.

Microscopic observation of tissues in paraffin shows that, when compared to implants of biomaterial alone, implants of biomaterial colonized by macrophages induce first an inflammation phenomenon, which is an important step to induce migration and homing of competent cells for tissue repair. A more important neovascularisation at the implantation site of biomaterial colonized with macrophages has also been observed.

The histological analysis of tissues in resin confirms the increase of neovascularisation for mice implanted with macrophages colonized biomaterials ; when compared to mice implanted only with biomaterial.

The therapeutic applications for tissue repair are confirmed in human bearing non healing ulcers. The ulcers covered with scaffold implanted with autologous macrophages show an improved cicatrization as measured by detersion and size of the ulcer.

CLAIMS

1 – Humanized biomaterial comprising a porous biocompatible composite material customized and implanted with monocyte derived cells and preferably with macrophages.

2 – Humanized biomaterial according to claim 1, wherein the biocompatible composite material is chosen among the following materials : microfibers, ceramic materials, metal oxides such as aluminum oxide, calcium phosphate ceramic, glass or carbon fibers, hydroxylapatite, silicon carbide or nitride, collagen polymers or a mixture of these different materials.

3 – Humanized biomaterial according to claims 1 or 2, wherein the human macrophages are liable to be obtained by *ex vivo* differentiation from blood monocytes leading to living macrophages, and are cultured under conditions enabling their penetration and adherence into the biomaterial, for instance for several hours at 37°C, with the porous biomaterial, allowing infiltration of the biomaterial and substantially irreversible binding of the living macrophages to the biomaterial, being humanized with patient's macrophages and ready for implantation.

4 – Living body-supporting implant, characterized by the fact that it comprises or consists of the humanized biomaterial according to any one of claims 1 to 3, and is preferably structured under the form of scaffold, tissue-supporting sponges, bone or cartilage.

5 – Use of a humanized biomaterial according to any one claims 1 to 3 or of a living body-supporting implant according to claim 4, for the preparation of a tissue implant destined to replace or repair defective tissue, such as defective bone, cartilage, dental tissue, fibrous tissue, fibrocartilaginous supporting tissue.

6 – Use of a humanized biomaterial according to any one of claims 1 to 3 or of a living body-supporting implant according to claim 4, wherein the monocyte derived cells or macrophages are autologous with respect to the tissue to be replaced or repaired, enabling the biomaterial or the living body-supporting implant to be recognized as self.

7 – Process for the preparation of a humanized biomaterial according to any one claims 1 to 3, comprising the following steps :

- preparation of the porous biomaterial structured in form of bones, cartilage,
- preparation of macrophages from blood monocytes,
- immersion of the biomaterial in a physiologic solution appropriate for the culture of macrophages which are added afterwards (ex. : phosphate buffered saline, medium such as RPMI, IMDM, AIMV),
- addition of the macrophages to the solution under conditions enabling binding to biomaterial and particularly for 1 to 20 h. at 37°C,
  5 % CO2 and 5 % air,
- washing of the biomaterial and conservation until use in physiologic medium.

8 – Process for the preparation of a living body-supporting implant according to claim 4, comprising the following steps :

- preparation of a customized porous implant or scaffold composed of bio-compatible material, according to any one of claims 1 to 3,
- preparation of macrophages from blood monocytes of the patient needing the customized implant of biomaterial,
- co-culture of macrophages and the implant in adequate medium under conditions enabling penetration and adherence to the biomaterial in particular at 37°C, 5% CO2 in hydrophobic bags or containers until grafting the implant.

9 – Use of the humanized biomaterial according to any one of claims 1 to 3 or of a living body-supporting implant according to claim 4, which can be implanted in a tissue, for the *in vitro* or *in vivo* or *ex vivo* delivery of factors chosen in the group of chemokines and/or monokines, and/or cytokines and/or growth factors, the factors released being useful for the local attraction of cells required for tissue growth (such as osteoblasts, chondrocytes, fibroblasts, epithelial cells.....) and/or for the neovascularization around the implanted biomaterial, and/or for the release of growth factors sustaining proliferation of cells and/or the growth of new tissues.

10 - Use of the humanized biomaterial according to any one of claims 1 to 3 or of a living body-supporting implant according to claim 4, as a graft for the replacement of supporting tissues such as bones, cartilages, dental tissues, epithelial sheet and subcutaneous tissue matrix.

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(54) Title: NEW HUMANIZED BIOMATERIALS, A PROCESS FOR THEIR PREPARATION AND THEIR APPLICATIONS

(57) Abstract: The invention relates to humanized biomaterial comprising a porous biocompatible composite material customized and implanted with monocyte derived cells preferably with macrophages.

Attorney Docket No. 0508-1001

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: HUMANIZED BIOMATERIALS, A PROCESS FOR THEIR PREPARATION AND THEIR APPLICATIONS

the specification of which: *(check one)*

REGULAR OR DESIGN APPLICATION

☐ is attached hereto.

☐ was filed on _____ as application Serial No. _____

and was amended on _____ (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

☒ was described and claimed in International application No. PCT/EP00/08157 filed on August 22, 2000 and as amended on _____(if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Country	Application Number	Date of Filing (day, month, year)	Priority Claimed
EUROPE	99402149.1	30 August 1999	Yes

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional patent application(s) listed below:

Application No.	Filing Date	Status (patented, pending abandoned)

(Complete this part only if this is a continuing application.)

I hereby claim the benefit under 35 USC 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status (patented, pending abandoned)

Docket No. 0508-1001

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 - CC

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